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B.J. Berger and M.H. Knodel
DRDC Suffield

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Principal Author

Bradley J. Berger

Approved by

L. P. Nagata

Head, Biotechnology Section

Approved for release by

P. A. D'Agostino

Chair, DRDC Suffield Document Review Committee

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# Abstract

Pyridinium oxime compounds have been utilised by a number of military organisations as one of the antidotes for nerve-agent poisoning. In Canada, the preferred compound from this class is HI-6 [1-[((4-carbomylpyridino)methoxy)methyl]-2-[(hydroxyimino)methyl]pyridinium dichloride or dimethanesulfonate], which has been demonstrated to be tolerated at high doses without significant ill effects. In this study, HI-6 and 15 structural analogues have been examined for their antimicrobial properties against a series of model organisms: *Bacillus cereus* and *B. anthracis* Sterne (as models for virulent *B. anthracis*), *Ochrobactrum intermedium* (as a model for *Brucella spp.*), *Mycobacterium marinum* (as a model for *M. tuberculosis*), and *Crithidia luciliae* (as a model for *Leishmania spp.*). In general, the compounds were found to have little to no antimicrobial effect, with KJD-2-11, a thiourea derivative, being the most active in all the test systems. This analogue had an $IC_{50}$ of 350 μM against *B. cereus* in a rich medium and 80 μM against *B. anthracis* Sterne in a minimal defined medium, 720 μM against *O. intermedium*, 28 μM against *M. marinum*, and 27 μM against *C. luciliae*. In contrast, HI-6 had an $IC_{50}$ of 69 μM against *M. marinum*, but had no detectable effect on any other organism up to the maximum tested concentration (1.0 or 10 mM). The results of this study indicate that the pyridinium oxime compounds already used as nerve-agent antidotes will have no antimicrobial effect against biological threat agents, and cannot be relied upon for additional protection in the event of a combined chemical-biological incident. The results also validate the utility of screening test compounds against biosafety level-1 and -2 model organisms prior to investing in screening against fully pathogenic biohazard safety level-3 agents.

# Résumé

Un certain nombre d'organismes militaires a utilisé les composés d'oxime de pyridinium comme antidote contre l'intoxication par agents neurotoxiques. Au Canada, le composé préféré de cette classe est l'antidote oxime HI-6 [1-[((4-carbomylpyridino)methoxy)methyl]-[(hydroxyimino)methyl]dichlorure de pyridinium ou diméthanesulfonate], prouvé être toléré à grandes doses sans effets nocifs importants. Cette étude examine les propriétés antimicrobiennes de l'oxime HI-15 et 14 analogues structuraux contre une série d'organismes d'étalonnage : *Bacillus cereus* et *B. anthracis* Sterne (comme modèles pour *B. anthracis* virulent), *Ochrobactrum intermedium* (comme modèle pour *Brucella spp.*), *Mycobacterium marinum* (comme modèle pour *M. tuberculosis*), et *Crithidia luciliae* (comme modèle pour les *Leishmania spp.*). On trouve, qu'en général, ces composés n'ont que peu ou aucun effet antimicrobien, avec KJD-2-11, un dérivé thiourée, comme étant le plus actif dans tous les systèmes d'essais. Cet analogue avait une $IC_{50}$ de 350 µM contre *B. cereus* dans un riche milieu et de 80 µM contre *B. anthracis* Sterne dans un milieu minimum, 720 µM contre *O. intermedium*, 28 µM contre *M. marinum*, et 27 µM contre *C. luciliae*. L'oxime HI-6 avait en contraste une $IC_{50}$ de 69 µM contre *M. marinimum* mais n'avait aucun effet détectable sur aucun autre organisme jusqu'à une concentration maximum testée (1,0 ou 10 mM). Les résultats de cette étude indiquent que les composés d'oxime de pyridinium déjà utilisés comme antidote contre les agents neurotoxiques n'auront pas d'effet antimicrobien contre les agents de menace biologiques et ne seront pas fiables comme protection supplémentaire en cas d'incident combinant les agents biologiques et chimiques. Les résultats valident aussi l'utilité d'effectuer des tests de dépistage des composants contre les organismes de biosécurité de niveaux 1 et 2 avant d'investir en un dépistage contre les agents pathogéniques ayant un biorisque de niveau 3.

# Executive summary

## Pyridinium Oxime Compounds as Antimicrobial Agents

**Bradley J. Berger; Marvin H. Knodel; DRDC Suffield TM 2007-176; Defence R&D Canada – Suffield; August 2007.**

## Background

Pyridinium oximes, such as 2-PAM, HI-6, and toxogonin, are a key component of the nerve-agent antidotes fielded by numerous militaries. For the Canadian Forces (CF), the multicomponent autoinjector contains HI-6 [1-[((4-carbomylpyridino)methoxy)methyl]-2-[(hydroxyimino) methyl]pyridinium dichloride or dimethanesulfonate] for reactivation of acetylcholinesterase inhibited by nerve-agent. HI-6 has been shown in a variety of studies to be very well tolerated at high doses, giving rise to the possibility that the high serum levels of HI-6 achievable in practice could have utility in protecting against other threats and/or diseases faced by the CF. One particular scenario of relevence would be a combined nerve-agent/biological-agent incident, where individuals would use HI-6 in response to the nerve agent component. In order to assess the potential antimicrobial properties of pyridinium oximes, HI-6 and 15 structural analogues were tested against a variety of model organisms for a variety of pathogens. *Bacillus cereus* and *B. anthracis* Sterne were used as models for virulent *B. anthracis*, *Ochrobactrum intermedium* as a model for *Brucella spp.*, *Mycobacterium marinum* as a model for *M. tuberculosis*, and *Crithidia luciliae* as a model for *Leishmania spp.* Any compounds found to have efficacy against the model organisms would then be tested against the fully pathogenic threat agents under appropriate containment conditions.

## Principal results

In general, the compounds were found to have little to no antimicrobial effect, with KJD-2-11, a thiourea derivative, being the most active in all the test systems. This analogue had a median effect concentration ($IC_{50}$; the dose required to suppress microbial growth by 50%) of 350 μM against *B. cereus* in a rich medium and 80 μM against *B. anthracis* Sterne in a minimal defined medium, 720 μM against *O. intermedium*, 28 μM against *M. marinum*, and 27 μM against *C. luciliae*. In contrast, HI-6 had an $IC_{50}$ of 69 μM against *M. marinum*, but had no detectable effect on any other organism up to the maximum tested concentration (1.0 or 10 mM). This lack of effect was also seen with the other pyridinium oximes used or considered in fielded nerve-agent antidotes: 2-PAM, toxogonin, and HLo-7.

## Significance of results

As a class, the pyridinium oximes displayed no appreciable antimicrobial activity. In particular, HI-6, as the compound of this class chosen for use in CF autoinjectors, was completely devoid of any ability to prevent microbial growth. Despite the high serum levels safely attainable by this compound, HI-6 would have no utility in the prophylaxis or treatment of the infectious diseases examined here. Use of the CF autoinjector during a combined nerve-agent/biological-agent

incident is unlikely to be beneficial for the biological agent, particularly if anthrax or *Brucella spp.* is the agent involved.

## Future Work

As the compounds tested in this study remain at DRDC Suffield, it is possible to test for antimicrobial activity against additional biothreat agents. In particular, there might be value in testing against malaria and/or viral agents. However, the present results suggest that further testing against bacterial pathogens is unlikley to uncover significant antimicrobial activity.

# Sommaire

## Pyridinium Oxime Compounds as Antimicrobial Agents

**Bradley J. Berger; Marvin H. Knodel; DRDC Suffield TM 2007-176; R & D pour la défense Canada – Suffield; août 2007.**

## Contexte

Les oximes de pyridinium, telles que 2-PAM, HI-6 et toxogonin sont les composants clés des antidotes d'agents neurotoxiques mis en service par de nombreux organismes militaires. En ce qui concerne les Forces canadiennes (FC), l'injecteur automatique à composants multiples contient l'antidote oxime HI-6 [1-[((4-carbomylpyridino)methoxy)methyl]-2-[(hydroxyimino) methyl]dichlorure de pyridinium ou diméthanesulfonate] pour la réactivation de l'acétylcholinestérase inhibée par l'agent. neurotoxique. Plusieurs études ont démontré que l'antidote oxime HI-6 était bien tolérée à doses élevées ce qui entraîne la possibilité que les hauts niveaux de sérum de l'oxime HI-6 réalisables en pratique pourraient avoir leur utilité dans la protection contre d'autres menaces et/ ou maladies auxquelles les FC doivent faire face. Un scénario d'une pertinence particulière serait un incident combinant les agents neurotoxiques aux agents biologiques durant lequel des individus utiliseraient l'antidote oxime HI-6 en réponse au composant neurotoxique. Pour être en mesure d'évaluer les propriétés antimicrobiennes potentielles des oximes de pyridinium, on a testé, l'antidote oxime HI-6 et 15 analogues structurels contre une variété d'organismes d'étalonnage, pour une variété de pathogènes. On a utilisé le *Bacillus cereus* et *B. anthracis* Sterne comme modèles pour *B. anthracis* virulent, *Ochrobactrum intermedium* comme modèle pour *Brucella spp.*, *Mycobacterium marinum* comme modèle pour *M. tuberculosis*, et *Crithidia luciliae* comme modèle pour *Leishmania spp.* Les composants ayant une efficacité contre les organismes modèles seront ensuite testés contre les agents pathogéniques de menace dans les conditions de confinement appropriées.

## Résultats principaux

On a trouvé, qu'en général, ces composés n'ont que peu ou aucun effet antimicrobien, avec KJD-2-11, un dérivé thiourée, comme étant le plus actif dans tous les systèmes d'essais. Cet analogue avait une concentration efficace moyenne ($IC_{50}$ la dose requise pour supprimer la croissance microbienne de 50%) de 350 µM contre *B. cereus* dans un riche milieu et de 80 µM contre *B. anthracis* Sterne dans un milieu minimum, 720 µM contre *O. intermedium*, 28 µM contre *M. marinum*, et 27 µM contre *C. luciliae*. L'oxime HI-6 avait en contraste une $IC_{50}$ de 69 µM contre *M. marinimum* mais n'avait pas d'effet détectable sur aucun autre organisme jusqu'à une concentration maximum testée (1,0 ou 10 mM). Ce manque de résultat avait été aussi observé avec les autres oximes de pyridinium utilisées ou considérées dans les antidotes mises service contre les agents neurotoxiques.

## Portée des résultats

La classe des oximes de pyridinium ne présente pas d'activité antimicrobienne appréciable. L'antidote oxime HI-6 en particulier, ayant été choisi comme le composant de cette classe dans les injecteurs automatiques FC, était complètement dépourvu de la capacité d'empêcher la croissance microbienne. Malgré les hauts niveaux de sérum réalisables par ce composé, l'oxime HI-6 n'aurait pas d'utilité dans la prophylaxie ou le traitement des maladies infectieuses examinées ici. L'utilisation de l'injecteur automatique durant un incident combinant les agents neurotoxiques et biologiques ne serait probablement pas efficace contre l'agent biologique, surtout s'il s'agit du charbon bactéridien ou *Brucella spp.*

## Travaux futurs

Les composants testés dans cette étude sont conservés à RDDC Suffield et il est donc possible de tester l'activité antimicrobienne contre d'autres agents biologiques. Il pourrait être particulièrement utile de tester contre la malaria et /ou des agents viraux. Les résultats actuels suggèrent cependant qu'il est peu probable que les essais ultérieurs contre des pathogènes bactériologiques révèlent une activité antimicrobienne importante.

# Table of contents

# List of figures



# List of tables

# Acknowledgements

The authors would like to thank Dr. Pierre Lecavalier and Dr. Paul Lundy for their help in finding the structural formulas for the compounds used in this study.

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# Introduction

Pyridinium oximes (Figure 1) have been demonstrated to be potent reactivators of organophosphate-inhibited acetylcholinesterase [1]. Numerous members of this class of compound have been investigated and selected analogues, such as 2-PAM [2-[(hydroxyimino)methyl]-1-methylpyridinium chloride], toxogonin [1,1'-[oxybis(methylene)]bis [4-(hydroxyimino)methyl]pyridinium dichloride], and HI-6 [1-[((4-carbomylpyridino) methoxy)methyl]-2-[(hydroxyimino)methyl]pyridinium dichloride or dimethanesulfonate] are fielded by the militaries of several nations in predosed autoinjectors for defence against nerve agent poisoning [1]. As a class, these compounds are well tolerated in humans and test animals at high doses [1], with up to 1899 mg HI-6 successfully administered to pigs intravenously [2]. Rather than toxicity, the major factor in determining the oxime of choice is the relative ability of the compound to reactivate acetylcolinesterase inhibited by various organophosphate nerve agents [1].

One of the major weaknesses of many, but not all, of the pyridinium oximes is the low stability in aqueous solution. In particular, HI-6, HS-6, and HLo-7 display a complex pattern of disassociation depending on the pH of the solution which can lead to the formation of hydrogen cyanide and/or hydroxyacetonitrile (Figure 2) [3-6]. For this reason, the compounds are resuspended immediately before administration.

As selected pyridinium oximes have been successfully administered to humans in relatively large doses (up to 500 mg HI-6 via intramuscular injection [7-10]), and the pharmacokinetics of these compounds are well documented [7-18], it would be of some interest to determine if the drugs had any effectiveness against biological disease agents of interest to the military. The existence at DRDC Suffield of a set of pyridinium oximes (Figure 1) would allow for a detailed structure-activity analysis of any antimicrobial properties. This paper details the results of an initial screen of a series of pyridinium oximes (and related compounds) against a number of lower pathogenicity model organisms. Use of model organisms in the first stage screening saves on the time and expense of BSL-3 studies, and allows for a determination of which compounds and BSL-3 organisms are worth further exploration.

In this paper, *Bacillus cereus* and *B. anthracis* Sterne vaccine strain are used as models for fully virulent *B. anthracis*, *Ochrobactrum intermedium* for *Brucella spp.*, *Mycobacterium marinum* for *M. tuberculosis*, and *Crithidia luciliae* for *Leishmania spp.* These model systems are close relatives to the pathogens of interest. *Ochrobactrum intermedium* (formerly specific strains of *Ochrobactrum anthropi*) is a soil bacterium that is the closest known relative of the *Brucella spp.* based on 16S rRNA sequence identity and significant serological cross-reactivity [19]. *O. intermedium* is listed as a BSL-1 organism in the United States, but is not explicitly categorised on the United Kingdom or Canadian organism lists. We have opted to work with *O. intermedium* at BSL-2 due to literature reports of cases *O. anthropi* infections in predominantly immunocompromised individuals [20-22]. *Mycobacterium marinum* is the causative agent of fish and amphibian tuberculosis, and is the closest relative of *M. tuberculosis* by 16S rRNA analysis that is not a member of the *M. tuberculosis* complex [23,24]. While classified as a slow-growing mycobacterium species, *M. marinum* grows significantly more rapidly than *M. tuberculosis* (days rather than weeks), which facilitates its use as a model organism. While *M. marinum* grows optimally at 30°C and cannot grow at 37°C, it can cause cutaneous granuloma infections in

humans and must be cultured under BSL-2 conditions. *Crithidia spp.* are monogenic trypanosomatids that infect a variety of insect species, and have been widely used for decades as an easily cultured model for trypanosomes and leishmania. Ribosomal RNA analysis has shown that *Crithidia spp.* are the closest known relative to *Leishmania spp.*, and thus can be considered an appropriate model for leishmania promastigotes [25,26]. All *Crithidia spp.* are BSL-1 organisms and *Crithidia luciliae* was utilised as it was being maintained in this laboratory for other purposes.

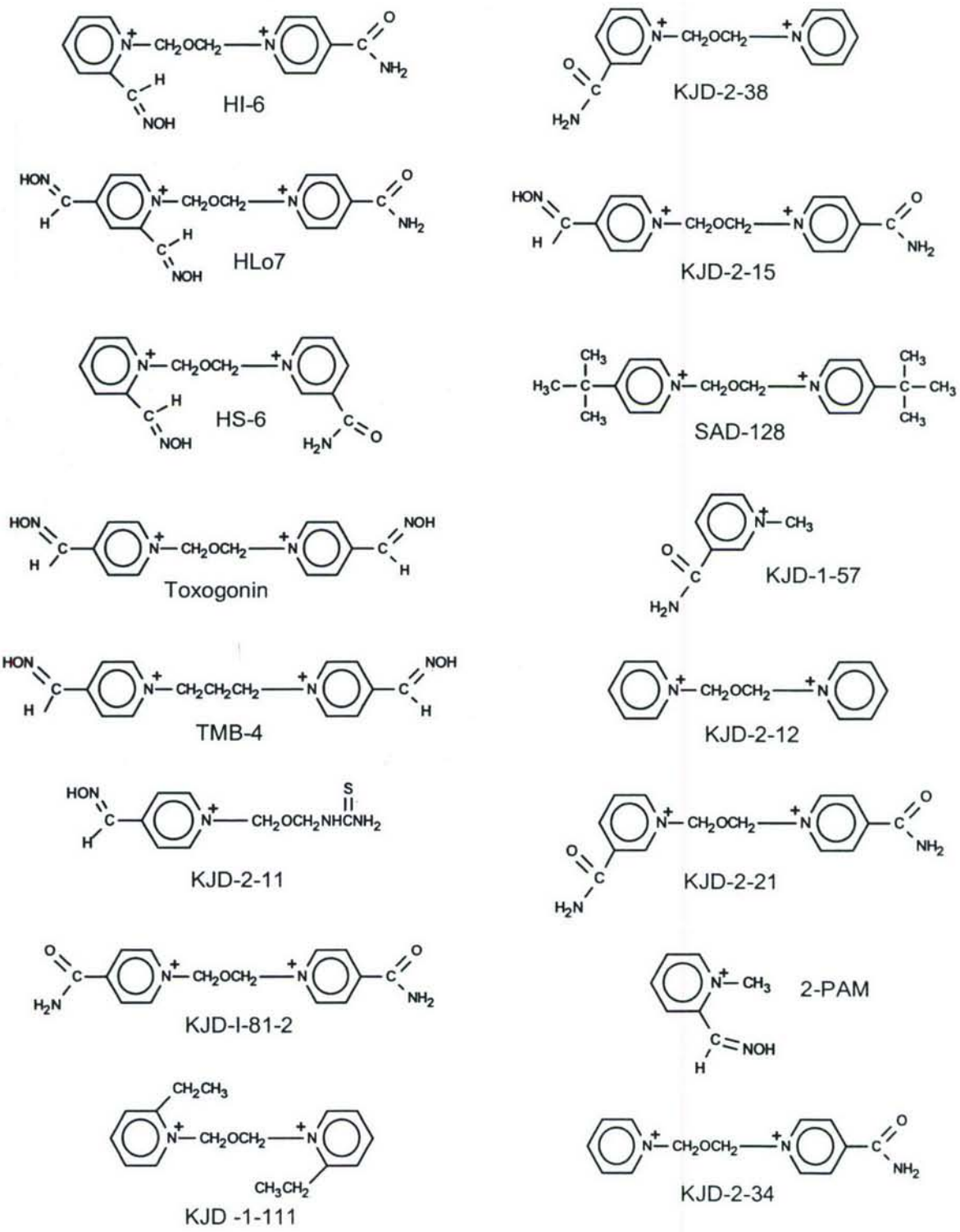


*Figure 1: Structures of the compounds used in the study.*

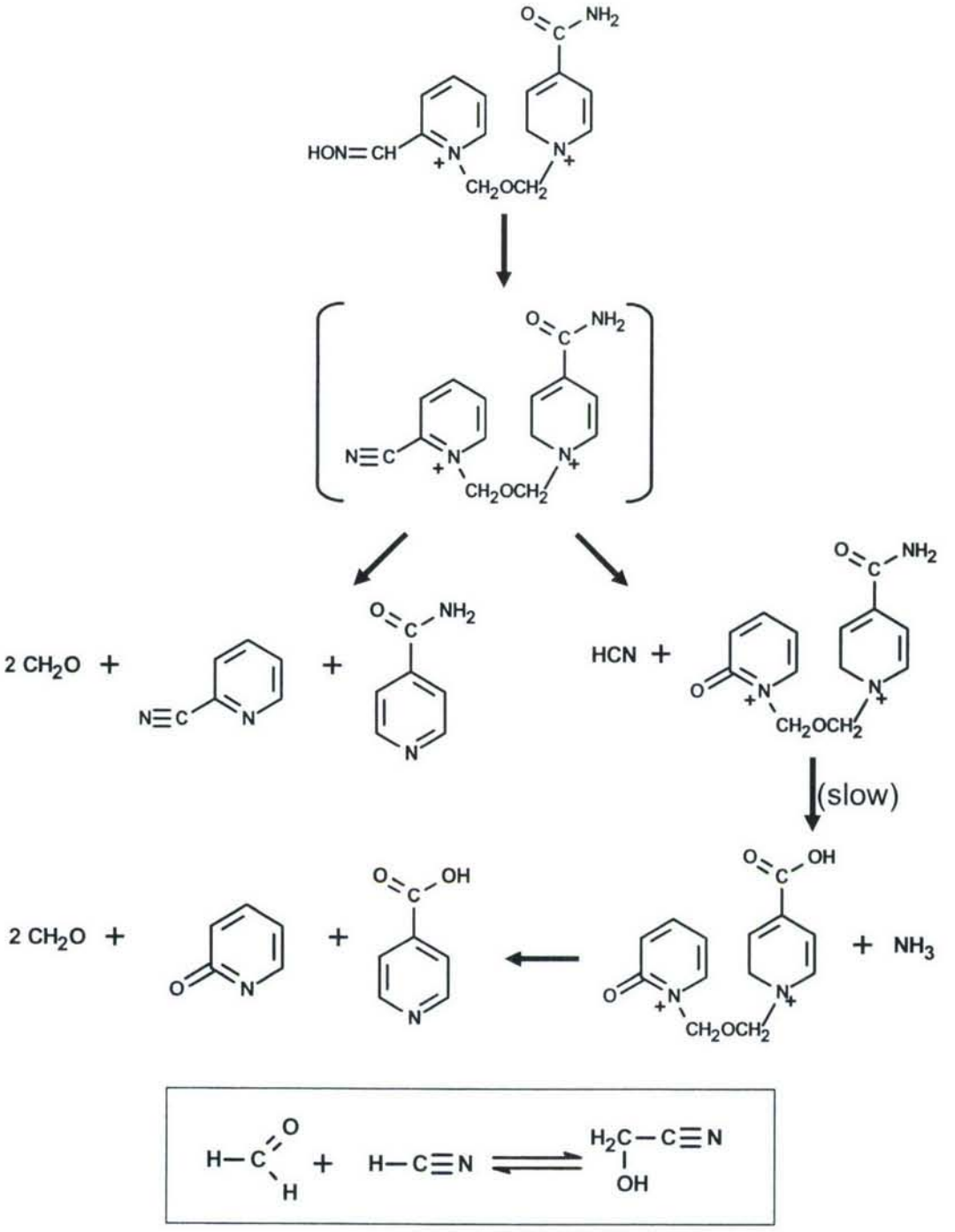


*Figure 2: The decomposition of bis-pyridinium oxime compounds.*

*The decomposition of HI-6 at physiological pH is shown as an example, with the inset showing the reaction of formaldehyde and hydrogen cyanide to form hydroxyacetonitrile. The data in the figure is adapted from Eyer et al. [4].*

# Materials and Methods

## Organisms

*Bacillus cereus* ATCC14579 was obtained form the American Type Culture Collection (Manassas, VA, USA) and was grown in Nutrient Broth in a shaking incubator (New Brunswick; Edison, NJ, USA) at 30°C and 250 rpm. *Bacillus anthracis* Sterne was obtained as a spore suspension from the Colorado Serum Co. (Denver, CO, USA) and grown in Nutrient Broth or defined medium at 37°C and 250 rpm. The defined medium used was a novel derivation of RS medium [27] that permits the growth of *B. cereus*, *B. anthracis*, or *B. thuringiensis* over numerous subcultures (data not shown), and consisted of 17.22 mM $K_2HPO_4$, 95.23 mM $NaHCO_3$, 50.34 µM $CaCl_2$, 82.25 µM $MgSO_4$, 5.32 µM $MnSO_4$, 0.5 mM iron ammonium citrate, 1 µM $CuSO_4$, 1 µM $ZnSO_4$, 1 µM $CoCl_2$, 1 µM $H_3BO_3$, 1 µM $(NH_4)_6Mo_7O_{24}$, 0.17 mM L-tryptophan, 0.87 mM glycine, 0.79 mM L-tyrosine, 1.57 mM L-lysine, 1.48 mM L-valine, 1.75 mM L-leucine, 1.30 mM L-isoleucine, 1.01 mM L-threonine, 1.38 mM L-aspartate, 4.16 mM L-glutamate, 0.37 mM L-proline, 0.35 mM L-histidine, 0.59 mM L-arginine, 0.76 mM L-phenylalanine, 2.24 mM L-serine, 0.3 mM L-methionine, 0.3 mM L-cystine, 2.96 µM thiamine, 12.49 µM uracil, 15.54 µM adenine, and 13.88 mM glucose (pH 7.4).

*Ochrobactrum intermedium* NCTC12171 (*Ochrobactrum anthropi* CNS2-75, LMG3301) was obtained from the National Centre for Type Cultures (London, UK) and was cultured in Brucella Broth (Becton Dickinson; Sparks, MD, USA) at 37°C and 250 rpm. *Mycobacterium marinum* NCTC2275 (ATCC927, TMC1218) was obtained from the National Centre for Type Cultures and grown in Middlebrook 7H9 complete medium with oleate/albumin/dextrose/catalase supplement at 30°C and 150 rpm. *Crithidia luciliae* ATCC30258 was obtained from the American Type Culture Collection and grown at 28°C in tissue culture flasks. The medium for *C. luciliae* was RPMI 1640 (containing 25 mM HEPES and 300 mg/L glutamine; Invitrogen, Burlington, ON, Canada) supplemented with 10 µg/mL folic acid, 5 µg/mL hemin, 40 U/mL penicillin/streptomycin (Invitrogen), 1 X MEM vitamins (Invitrogen), and 20 µg/mL adenosine. Unless otherwise stated, chemicals and media were obtained from Sigma/Aldrich (Oakville, ON, Canada).

## Antimicrobial Assay

Test compounds were resuspended to 20 mM or 2 mM in culture medium, filter sterilised, and 100 µl added to 96-well microtitre plates to yield 12-fold doubling dilutions. Bacterial cultures in mid-log growth were diluted to to 2 x $10^5$ cfu/mL in culture medium and 100 µl added to the microtitre plates. The final concentration of the test compounds ranged from 10 mM – 4.9 µM or 1.0 mM – 490 nM. Positive and negative growth controls were performed by replacing the inhibitor or the inoculum with 100 ul of culture medium respectively. The controls were performed at a replicate of n = 12, while each concentration of inhibitor was tested at a replicate of n = 6. The microtitre plates were sealed and incubated with no agitation at 37°C (*B. anthracis*, *O. intermedium*), 30°C (*B. cereus*, *M. marinum*), or 28°C (*C. luciliae*) for 24 hours (*B. anthracis*, *B. cereus*, *O. intermedium*) or 7 days (*C. luciliae*, *M. marinum*). Growth in all bacterial samples was then measured by $A_{650nm}$ using a Molecular Devices VersaMax 96-well spectrophotometer (Sunnyvale, CA, USA). Growth in the crithidial samples was measured by the method of

Zinsstag et al. [28] which monitors acidification of the culture medium, and the resulting yellow colour change of phenol red, at $A_{570nm}$ and $A_{405nm}$. The MIC was determined as the lowest dilution of test compound that completely prevented microbial growth, while the $IC_{50}$ was determined by non-linear curve fitting with the Scientist software package (MicroMath; Salt Lake City, UT, USA) programmed with the median dose equation [29].

For *M. marinum* only, several compounds were also assayed in separate, sealed tubes rather than microtitre plates. In these cases, test compounds were resuspended to 2 mM in Middlebrook 7H9 complete medium and serially diluted 10-fold. One mL of diluted test compound was mixed with 1.0 mL of 2 x $10^5$ cfu/mL *M. marinum* in the same culture medium in sterile 15 mL tubes. The final concentration of the test compounds ranged from 1.0 mM – 100 nM. Positive and negative growth controls were performed by substituting the inhibitor or inoculum with 1.0 mL of culture medium. Both controls and test samples were performed at a replicate of n = 3. The tubes were then sealed and incubated at 30°C and 150 rpm agitation for 4 days. Growth was measured at $A_{650nm}$ using a Pharmacia Ultrospec 1000 cuvette spectrophotometer (Baie D'Urfe, QB, Canada). MIC and $IC_{50}$ values were determined as above.

# Results and Discussion

## *Bacillus cereus* and *B. anthracis*

The pyridinium oximes were initially tested against *B. cereus* in a rich culture medium, and were found to have very little antibacterial effect (Table 1). Only one compound, KJD-I-81-2, was able to completely inhibit the growth of *B. cereus* at 10 mM. The best compound on the basis of the $IC_{50}$ value was KJD-2-11, at 350 μM. However, this drug still permitted some residual growth even up to 10 mM. In general, the compounds were unable to exert much antibacterial activity, with the maximum growth inhibition being below 50% at 10 mM for 13 of the 16 compounds.

*Table 1: The antimicrobial effect of HI-6 analogues against Bacillus cereus*

*The compounds were tested as outlined in the Materials and Methods section. The MIC is the lowest dilution that completely inhibits microbial growth, the $IC_{50}$ is the concentration calculated to cause 50% growth inhibition, and the maximal growth inhibition reflects that seen at the highest concentration tested.*

| Compound | MIC | $IC_{50}$ | Maximum Growth Inhibition (%) |
|---|---|---|---|
| HI-6 | >10 mM | >10 mM | 35.91 ± 3.12 |
| HLo-7 | >10 mM | >10 mM | 38.65 ± 0.96 |
| HS-6 | >10 mM | >10 mM | 44.71 ± 2.11 |
| 2-PAM | >10 mM | >10 mM | 16.00 ± 0.63 |
| Toxogonin | >10 mM | >10 mM | 13.07 ± 1.43 |
| TMB-4 | >10 mM | >10 mM | 10.34 ± 0.85 |
| KJD-2-11 | >10 mM | 349.20 ± 43.46 μM | 91.25 ± 1.51 |
| KJD-I-81-2 | 10 mM | 5.70 ± 0.93 mM | 100 |
| KJD-2-21 | >10 mM | 6.96 ± 1.56 mM | 65.45 ± 2.11 |
| KJD-2-38 | >10 mM | >10 mM | 32.40 ± 3.30 |

| | | | |
|---|---|---|---|
| KJD-2-15 | >10 mM | >10 mM | 25.36 ± 3.85 |
| KJD-1-111 | >10 mM | >10 mM | 19.19 ± 7.45 |
| SAD-128 | >10 mM | >10 mM | 10.36 ± 3.08 |
| KJD-1-57 | >10 mM | >10 mM | 5.70 ± 2.06 |
| KJD-2-12 | >10 mM | >10 mM | 5.12 ± 12.09 |
| KJD-2-34 | >10 mM | >10 mM | 20.90 ± 0.86 |

During the course of this investigation, DRDC Suffield obtained permission to derogate *B. anthracis* Sterne strain to BSL-2 containment. This alteration now allowed for a better model system for fully pathogenic anthrax. As we have previously seen that antimicrobial activity of test compounds can be higher against *Bacillus spp.* in a less rich, defined medium [30], *B. anthracis* Sterne was tested in a modified RS medium. In this medium, the compounds were indeed significantly more potent, with 7 drugs completely inhibiting bacterial growth. Both KJD-2-11 and KJD-I-81-2 had an MIC as low as 125 μM, and TMB-4, KJD-2-11, KJD-2-34 and KJD-I-81-2 all had $IC_{50}$ values below 100 μM. This obvious increase in antibacterial activity is interesting, as the modified RS medium differs from Nutrient Broth most notably in having free amino acids as opposed to proteins, and glucose as opposed to a more complex mixture of carbohydrates. It is possible that the pyridinium oximes bind to the proteins present in the rich medium, thus lowering the effective concentration of drug. Alternatively, the rich medium may contain a compound which competitively inhibits uptake of the drug. Finally, it is possible that growth in the defined medium may lead to the expression of a gene product which is the cellular target of the drug. However, the relevence of the differential effect of the oximes in these media to any clinical utility of the compounds against anthrax is unclear, although the in vivo environment is more likely reflected by the rich medium than the defined one.

*Table 2: The antimicrobial effect of HI-6 analogues against Bacillus anthracis Sterne*

*The compounds were tested as outlined in the Materials and Methods section. The MIC is the lowest dilution that completely inhibits microbial growth, the $IC_{50}$ is the concentration calculated to cause 50% growth inhibition, and the maximal growth inhibition reflects that seen at the highest concentration tested.*

| Compound | MIC | $IC_{50}$ | Maximum Growth Inhibition (%) |
|---|---|---|---|
| HI-6 | >1 mM | 168.05 ± 79.55 µM | 80.58 ± 13.33 |
| HLo-7 | >1 mM | 545.05 ± 205.93 µM | 71.64 ± 7.59 |
| HS-6 | >1 mM | 280.48 ± 53.24 µM | 81.05 ± 14.25 |
| 2-PAM | >1 mM | 160.01 ± 64.37 µM | 87.20 ± 14.78 |
| Toxogonin | >1 mM | >1 mM | 13.01 ± 6.57 |
| TMB-4 | 1 mM | 35.35 ± 12.12 µM | 100 |
| KJD-2-11 | 125 µM | 80.24 ± 2.90 µM | 100 |
| KJD-I-81-2 | 125 µM | 41.78 ± 1.80 µM | 100 |
| KJD-2-21 | 250 µM | 113.39 ± 1.41 µM | 100 |
| KJD-2-38 | 500 µM | 286.15 ± 72.80 µM | 100 |
| KJD-2-15 | >1 mM | 638.55 ± 50.36 µM | 76.39 ± 3.74 |
| KJD-1-111 | 250 µM | 108.05 ± 2.38 µM | 100 |
| SAD-128 | >1 mM | 171.11 ± 20.37 µM | 90.37 ± 7.56 |
| KJD-1-57 | >1 mM | 707.62 ± 151.97 µM | 62.37 ± 14.07 |
| KJD-2-12 | >1 mM | 333.89 ± 84.07 µM | 70.13 ± 19.64 |
| KJD-2-34 | 250 µM | 77.20 ± 3.43 µM | 100 |

## *Ochrobactrum intermedium*

As a closely related organism, *O. intermedium* was used as a model system for *Brucella spp.* in testing the pyridinium oximes. When used in a rich growth medium, Brucella Broth, the compounds were found to have very little antibacterial effect. The observed growth inhibition was similar to that seen against *B. cereus* grown in Nutrient Broth, with no compound able to completely prevent microbial growth. KJD-2-11 was found to be the best drug, with an $IC_{50}$ of 720 μM and 91% growth inhibition at 10 mM. No other compound had an $IC_{50}$ below 1 mM, although KJD-2-21 was able to inhibit 92% of bacterial growth at 1 mM. The overall results suggest that the compounds performed approximately twice as poorly against the gram-negative *O. intermedium* than against the gram-positive *B.cereus* when both were grown in rich media.

*Table 3: The antimicrobial effect of HI-6 analogues against Ochrobactrum intermedium*

*The compounds were tested as outlined in the Materials and Methods section. The MIC is the lowest dilution that completely inhibits microbial growth, the $IC_{50}$ is the concentration calculated to cause 50% growth inhibition, and the maximal growth inhibition reflects that seen at the highest concentration tested.*

| Compound | MIC | $IC_{50}$ | Maximum Growth Inhibition (%) |
|---|---|---|---|
| HI-6 | >10 mM | >10 mM | 36.59 ± 2.05 |
| HLo-7 | >10 mM | 1.67 ± 0.19 mM | 76.09 ± 14.83 |
| HS-6 | >10 mM | >10 mM | 48.43 ± 13.62 |
| 2-PAM | >10 mM | >10 mM | 11.43 ± 11.41 |
| Toxogonin | >10 mM | >10 mM | 5.47 ± 10.16 |
| TMB-4 | >10 mM | >10 mM | 20.68 ± 6.19 |
| KJD-2-11 | >10 mM | 716.04 ± 180.85 μM | 90.78 ± 0.42 |
| KJD-I-81-2 | >10 mM | 3.02 ± 0.20 mM | 70.61 ± 0.91 |
| KJD-2-21 | >10 mM | 2.72 ± 0.89 mM | 92.90 ± 0.98 |
| KJD-2-38 | >10 mM | >10 mM | 21.67 ± 2.78 |
| KJD-2-15 | >10 mM | >10 mM | 4.54 ± 6.73 |

| | | | |
|---|---|---|---|
| KJD-1-111 | >10 mM | >10 mM | 24.68 ± 3.18 |
| SAD-128 | >10 mM | >10 mM | 21.37 ± 5.11 |
| KJD-1-57 | >10 mM | >10 mM | 18.18 ± 2.92 |
| KJD-2-12 | >10 mM | >10 mM | 10.87 ± 6.62 |
| KJD-2-34 | >10 mM | >10 mM | 5.39 ± 0.98 |

## *Mycobacterium marinum*

*M. marinum* is closely related to *M. tuberculosis*, but has the advantage of much faster growth and substantially lower pathogenicity. As such, it is useful as a primary screening surrogate for antimycobacterial activity. As seen for the other bacteria examined above, many of the test compounds had extremely poor antimicrobial activity in this system. Only three of the compounds tested in 96-well plates were able to completely inhibit cell growth, with MIC values of 10 mM and $IC_{50}$ values of 1.5 – 6.0 mM.

An unusual phenomenon was observed with the *M. marinum* assays for HI-6, HLo-7, HS-6, and KJD-2-11 that had not been previously seen in any of the microtitre antibacterial assays. These four compounds repeatedly (n = 6), in experiments performed on different days with different batches of medium, gave an unusual growth pattern for the positive controls (Figure 3). Rather than consistent bacterial growth in all 12 control wells (A1 – A12 in the 96-well plates), growth ranged from normal in A12 downward to nothing in A1. The wells containing the test compounds (C-H1 – C-H12) also displayed the same pattern. After rigid controlling for any feature in the experimental setup which could cause any variation in cell growth, we came to the conclusion that the higher concentrations of drug (C-H1 contained 10 mM for example) might be liberating a volatile compound that was killing the cells. As mentioned in the Introduction section, HI-6, HLo-7, and HS-6 are known from the literature to break down in aqueous solution yielding formaldehyde and hydrogen cyanide (Figure 2). Both of these latter compounds are known volatile antimicrobials. However, it should be pointed out that this growth phenomenon was not seen in any of the other microbial test systems used in this study. The reason for this inhibition being seen only with *M. marinum* is due to, in our opinion, the presence of 4.0 μg/mL catalase in Middlebrook 7H9 complete medium. The catalase could be promoting the rapid breakdown of the oximes or could be disturbing the formation of hydroxyacetonitrile. Both of these events would have the consequence of increasing the amount of formaldehyde and cyanide present in the test plate.

In order to circumvent the growth issues with these four oximes, we altered the experimental procedure in order to isolate each individual sample from any potential volatile antimicrobial produced by the higher concentrations assayed. A smaller number of drug dilutions were tested in sealed 15 mL tubes and growth individually monitored. Under these conditions, growth inhibition was not seen in the positive control samples. All four oximes were found to have an MIC of 1 mM and IC50 values ranging from 28 μM for KJD-2-11 to 111 μM for HLo-7.

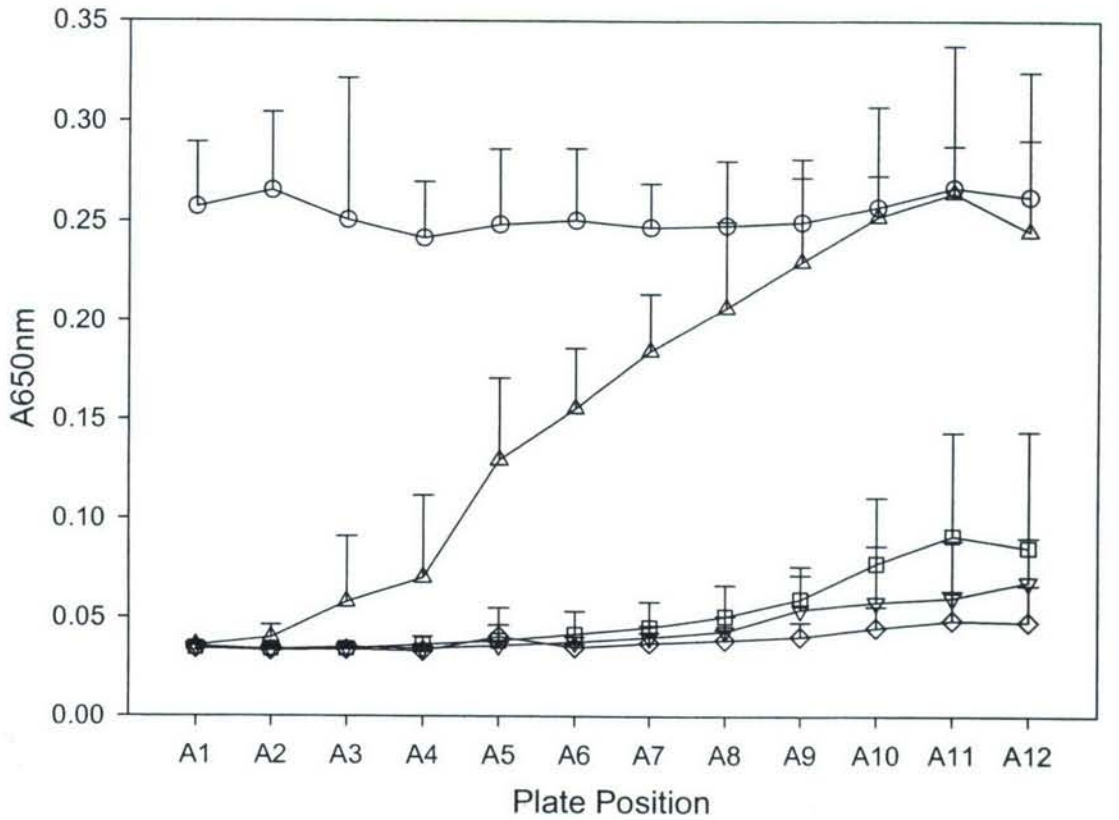


*Figure 3: An unusual growth pattern for positive control samples.*

*The turbidity at 650nm is shown for positive growth control samples of M. marinum in microtitre plates containing pyridinium oximes in other wells. The triangles represent control growth in the plates containing KJD-2-11, the squares the plates containing HI-6, the inverted triangles the plates containing HS-6, and the diamonds the plates containing HLo-7 (each data set is the mean and standard deviation of 4 separate plates). The circles represent the control growth across all the remaining plates (12 compounds, one plate each). The X-axis shows the plate position, where 10 mM test compound would be present in wells C1-H1, 5 mM in wells C2-H2, with subsequent 2-fold dilution through to C12-H12.*

*Table 4: The antimicrobial effect of HI-6 analogues against Mycobacterium marinum*

*The compounds were tested as outlined in the Materials and Methods section. The MIC is the lowest dilution that completely inhibits microbial growth, the $IC_{50}$ is the concentration calculated to cause 50% growth inhibition, and the maximal growth inhibition reflects that seen at the highest concentration tested.*

| Compound | MIC | $IC_{50}$ | Maximum Growth Inhibition (%) |
|---|---|---|---|
| HI-6* | 1 mM | 69.24 ± 3.48 µM | 100 |
| HLo-7* | 1 mM | 110.63 ± 0.77 µM | 100 |
| HS-6* | 1 mM | 60.77 ± 4.60 µM | 100 |
| 2-PAM | 10 mM | 1.45 ± 0.40 mM | 100 |
| Toxogonin | >10 mM | >10 mM | 45.40 ± 5.71 |
| TMB-4 | >10 mM | >10 mM | 16.37 ± 7.53 |
| KJD-2-11* | 1 mM | 27.62 ± 0.52 µM | 100 |
| KJD-I-81-2 | 10 mM | 4.82 ±1.15 mM | 100 |
| KJD-2-21 | 10 mM | 6.08 ± 1.10 mM | 100 |
| KJD-2-38 | >10 mM | >10 mM | 18.91 ± 14.52 |
| KJD-2-15 | >10 mM | >10 mM | 43.65 ± 4.10 |
| KJD-1-111 | >10 mM | >10 mM | 3.37 ± 13.41 |
| SAD-128 | >10 mM | >10 mM | 49.72 ± 12.15 |
| KJD-1-57 | >10 mM | >10 mM | 12.40 ± 14.89 |
| KJD-2-12 | >10 mM | >10 mM | 13.84 ± 9.46 |
| KJD-2-34 | >10 mM | >10 mM | 3.46 ± 10.29 |

*These compounds were tested in individual 15 mL tubes instead of 96-well microtitre plates.

## *Crithidia luciliae*

As *C. luciliae* was being cultured at the time in the laboratory for other purposes, and the organism is closely related to the human pathogens found in the genus *Leishmania*, the pyridinium oximes were tested against this protozoan. As shown in Table 5, only two of the compounds displayed any appreciable activity against *C. luciliae*. KJD-2-11 was found to be the most effective compound, with an MIC of 62.5 μM and a calculated $IC_{50}$ of 27 μM. KJD-2-21 was the only other oxime with any activity, with an MIC of 500 μM and an $IC_{50}$ of 330 μM. The complete lack of activity by HI-6, HS-6, and HLo-7 when compared to the bacterial test systems suggests that *Crithidia* either do not take up the compounds or lack the molecular target found in bacteria.

*Table 5: The antimicrobial effect of HI-6 analogues against Crithidia luciliae*

*The compounds were tested as outlined in the Materials and Methods section. The MIC is the lowest dilution that completely inhibits microbial growth, the $IC_{50}$ is the concentration calculated to cause 50% growth inhibition, and the maximal growth inhibition reflects that seen at the highest concentration tested.*

| Compound | MIC | $IC_{50}$ | Maximum Growth Inhibition (%) |
|---|---|---|---|
| HI-6 | >1 mM | >1 mM | 12.36 ± 2.83 |
| HLo-7 | >1 mM | >1 mM | 0.00 ± 5.90 |
| HS-6 | >1 mM | >1 mM | 0.00 ± 3.80 |
| 2-PAM | >1 mM | >1 mM | 3.98 ± 4.36 |
| Toxogonin | >1 mM | >1 mM | 1.32 ± 2.11 |
| TMB-4 | >1 mM | >1 mM | 9.04 ± 6.76 |
| KJD-2-11 | 62.5 μM | 27.32 ± 0.54 μM | 100 |
| KJD-I-81-2 | >1 mM | >1 mM | 24.23 ± 5.32 |
| KJD-2-21 | 500 μM | 332.11 ± 13.05 μM | 100 |
| KJD-2-38 | >1 mM | >1 mM | 20.88 ± 7.49 |
| KJD-2-15 | >1 mM | >1 mM | 21.29 ± 7.23 |

| | | | |
|---|---|---|---|
| KJD-1-111 | >1 mM | >1 mM | 31.37 ± 7.28 |
| SAD-128 | >1 mM | >1 mM | 16.37 ± 5.89 |
| KJD-1-57 | >1 mM | >1 mM | 26.00 ± 4.49 |
| KJD-2-12 | >1 mM | >1 mM | 33.99 ± 5.01 |
| KJD-2-34 | >1 mM | >1 mM | 14.31 ± 10.68 |

# Conclusions

While the set of pyridinium oxime compounds tested here varied in structure in such a manner as to allow for a comprehensive examination of structure-activity relationships, the class of compound was almost devoid of any significant antimicrobial properties. None of the compounds demonstrated a low μM MIC against any of the microbes examined, and no submicromolar $IC_{50}$ values were obtained. Many of the oximes displayed a complete lack of effect up to 10 mM against a broad variety of organism. Therefore, a detailed, meaningful analysis of structure-activity relationships was not possible. The best compound against all the test organisms was KJD-2-11, with $IC_{50}$ values ranging from 27 – 700 μM. It is interesting to note that this compound was the only one in the set with a ring substituent other than an aldoxime, carboxamide, or ethyl/t-butyl. Therefore, the antimicrobial properties seen with KJD-2-11 most likely are due to the presence of the thiourea moiety.

HI-6 and several other of the oximes examined are known to be tolerated in high doses in humans and have been used by the military for years [1,7-10]. It was anticipated that the ability for safely achieving high serum concentrations of these compounds would allow for a significant antimicrobial effect against organisms of interest to the Canadian Forces. In particular, it was hoped that HI-6 might be of particular advantage in the event of a combined nerve-agent/biological-agent incident. Unfortunately, the compounds appear to be tolerated as well by bacteria and protozoa as seen for mammals. Therefore, the utility of this class of compound does not extend to biological agents.

The failure of the pyridinium oximes as antimicrobials serves to underline the validity of our experimental approach. Screening of the compounds first using BSL-2 in vitro models is both time and cost effective when compared with all screening being performed in BSL-3 on fully pathogenic agents. In this particular case, several weeks of BSL-3 time has been left available for other projects by not testing directly on BSL-3 models. Should any of the test compounds shown promise in any of the BSL-2 models, then those particular compounds would be screened against the appropriate BSL-3 agent. It is recommended that any future novel therapeutic agents follow this testing procedure where appropriate model systems are available.

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# List of symbols/abbreviations/acronyms/initialisms

| | |
|---|---|
| CF | Canadian Forces |
| MIC | Minimal Inhibitory Concentration |
| $IC_{50}$ | Median Effect Concentration (50% Growth Inhibition) |
| BSL | Biohazard Safety Level |

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Pyridinium oxime compounds have been utilised by a number of military organisations as one of the antidotes for nerve-agent poisoning. In Canada, the preferred compound from this class is HI-6 [1-[((4-carbomylpyridino)methoxy)methyl]-2-[(hydroxyimino)methyl]pyridinium dichloride or dimethanesulfonate], which has been demonstrated to be tolerated at high doses without significant ill effects. In this study, HI-6 and 15 structural analogues have been examined for their antimicrobial properties against a series of model organisms: *Bacillus cereus* and *B. anthracis* Sterne (as models for virulent *B. anthracis*), *Ochrobactrum intermedium* (as a model for *Brucella spp.*), *Mycobacterium marinum* (as a model for *M. tuberculosis*), and *Crithidia luciliae* (as a model for *Leishmania spp.*). In general, the compounds were found to have little to no antimicrobial effect, with KJD-2-11, a thiourea derivative, being the most active in all the test systems. This analogue had an $IC_{50}$ of 350 μM against *B. cereus* in a rich medium and 80 μM against *B. anthracis* Sterne in a minimal defined medium, 720 μM against *O. intermedium*, 28 μM against *M. marinum*, and 27 μM against *C. luciliae*. In contrast, HI-6 had an $IC_{50}$ of 69 μM against *M. marinum*, but had no detectable effect on any other organism up to the maximum tested concentration (1.0 or 10 mM). The results of this study indicate that the pyridinium oxime compounds already used as nerve-agent antidotes will have no antimicrobial effect against biological threat agents, and cannot be relied upon for additional protection in the event of a combined chemical-biological incident. The results also validate the utility of screening test compounds against biosafety level-1 and -2 model organisms prior to investing in screening against fully pathogenic biohazard safety level-3 agents.

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[HI-6, Oximes, antimicrobial agents]

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